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Double replica technique applied to choroid plexus from early foetal sheep: completeness and complexity of tight junctions

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Summary

Choroid plexuses from early and late sheep foetuses were examined by an improved freeze-fracture technique and the use of double-replicas to define the structure of the tight junction network of the epithelial cells. 'Complex' strands which consist of two normal parallel strands separated by a single row of pits or particles are defined and demonstrated in complementary faces. Since this strand variety was encountered in the same proportion in the different developmental stages investigated, it could not be correlated with changes in permeability. It is more likely that the 'complex' strands are associated with the transfer of gap junction particles within the membrane. The question of the significance of discontinuities in P face ridges was also resolved by the double replica technique: the few discontinuities which were observed could be accounted for by particles in the complementary E faces. Furthermore, approximately the same number of such junction displacements was found in early and late stages which makes it unlikely that this phenomenon could contribute to changes in permeability. Thus it has not been possible so far to relate any structural feature of the tight junction network in developing choroid plexus epithelial cells with the reported changes in permeability of the blood–C.S.F. barrier.

Introduction

Recent studies of ultrastructure of the choroid plexus during brain development have suggested that the characteristic freeze-fracture appearance of tight junctions in this epithelium is present very early and remains unchanged throughout development in spite of large changes in the permeability of the blood–C.S.F. barrier during the same period of development (Dziegielewska *et al.*, 1979). The important features of epithelial cell tight junctions that have previously been associated with the degree of permeability of an epithelium are the strand number and to a lesser degree the junctional depth (Claude and Goodenough, 1973). However, in foetal sheep choroid plexus extensive measurements of these parameters

showed that neither changed significantly between 40 days gestation and 125 days gestation (term is 147 days) (Møllgård *et al.*, 1976). Two technical problems were encountered in this work: (1) some junctional strands appeared to be more than twice the width of the usual strands; and (2) in some replicas very small discontinuities could be seen in the P face ridges. Since occasional particles were seen in the grooves on the E face, it was thought that incomplete separation of the P face ridges may have occurred occasionally during the freeze-fracture process. An alternative explanation is of course that a discontinuity represents an absence of junction material and thus indicates a genuine possible pathway across one strand of the junctional complex.

We should like to present evidence from the use of the double replica technique that the wide 'double' strands are in fact made up of the normal parallel strands separated by a single row of pits or particles. Since these wide strands are more than a mere duplication of normal strands we propose to refer to them as 'complex' strands.

At all gestational ages investigated the proportion of 'complex' strands to single normal strands was virtually the same; thus the presence or absence of 'complex' strands cannot be correlated with the observed marked changes in permeability. Gap junction particles were, however, frequently completely sequestered within compartments of the tight junction network, which were connected to other gap junction-containing compartments via 'complex' strands. This suggests that the 'complex' strands might be associated with the distribution of gap junction particles during development.

The double replica technique also allowed us to resolve the problem of incomplete P face ridges; the interruptions can be accounted for by particles in the grooves of the complementary E faces.

Material and methods

The material examined comprises choroid plexus from adult sheep and foetal sheep of various gestational ages (30, 45, 60, 75 and 125 days).

A detailed description of the preparation of ewes and foetuses has been given (Dziegielewska *et al.*, 1979). Perfusion and immersion fixation were performed with Karnovsky's fixative as described earlier (Møllgård and Saunders, 1975) or with 3% glutaraldehyde in 0.1 M phosphate buffer. Pieces of choroid plexus were isolated 1 h after fixation *in situ* and then kept for another 5 h in the fixative. After a rinse in buffer the specimens were infiltrated with 25% buffered glycerol for 1–2 h and frozen in liquid Freon 22 cooled by liquid nitrogen. The material was then fractured and replicated in a Balzer's freeze-etch unit (BAF 301) equipped with a device to yield complementary membrane fracture faces, an electron beam gun and a quartz thin film monitor. After tissue digestion with chrome–sulphuric acid and cleaning, replicas were examined in a Hitachi HS8 electron microscope.

Results

The junctional (or presumptive junctional) region of choroid plexus epithelial cells is well defined because the epithelium, even from the earliest specimen studied,

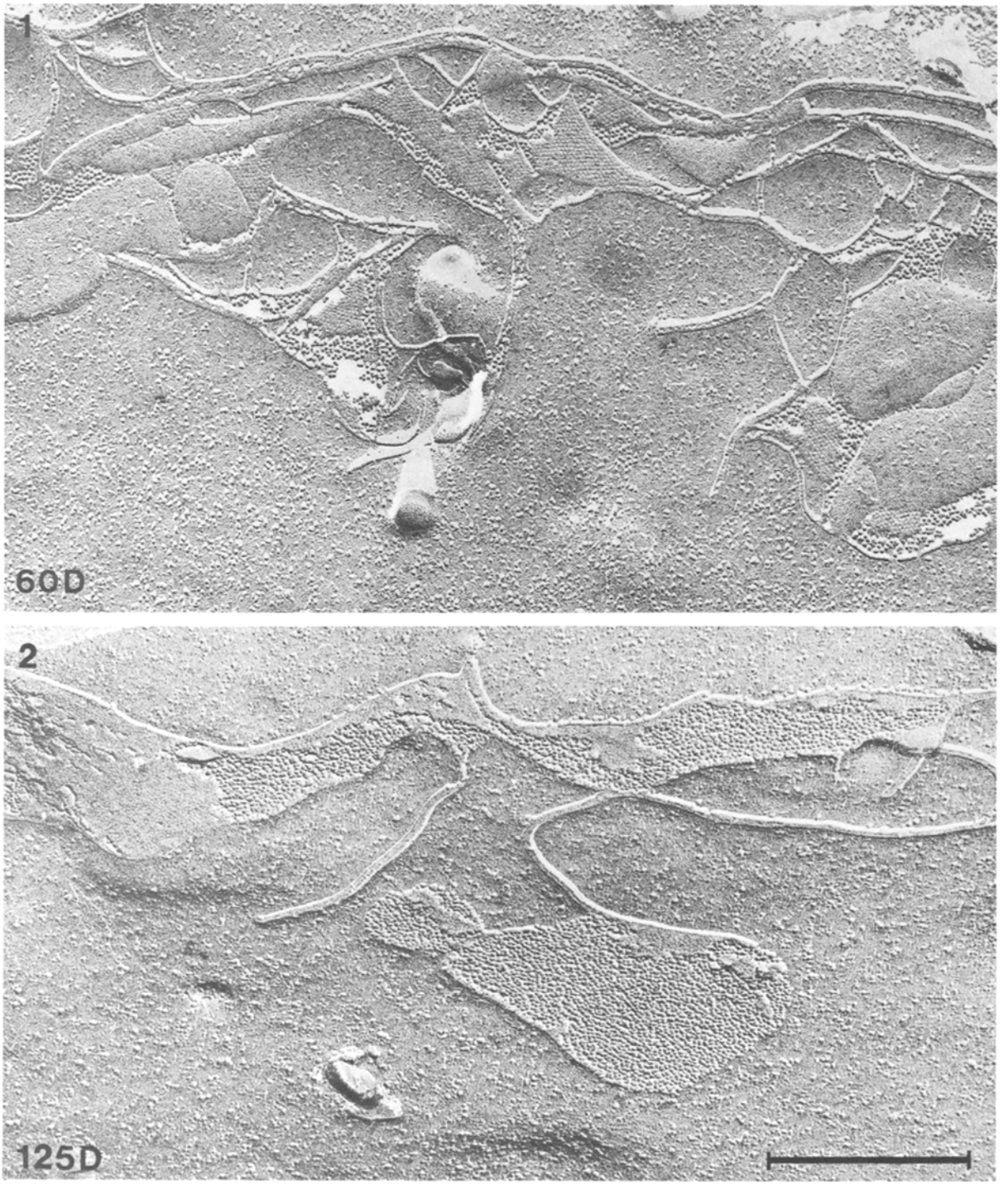
exhibits characteristic microvilli which define the apical part of the lateral cell membrane. Freeze-fractured non-junctional membranes of early choroidal epithelial cells reveal the usual distribution of intramembranous particles. The tight junction network seems to form a complete belt-like structure around each choroidal epithelial cell since it is present whenever the apical part of the lateral cell membrane is exposed during the freeze-cleavage.

So far more than 1000 junctions from 30 days gestation to adult life have been analysed. These data confirm our previous reports on the presence of complex tight junctions between choroid plexus epithelial cells from very early on in gestation (for references, see Introduction). The published quantitative data will not be commented upon, but the use of high resolution replicas and the double replica technique has allowed us to probe further into the fine construction of the junctional network.

The characteristic freeze-fracture morphology of tight junctions from choroid plexus epithelial cells is shown in Figs. 1 and 2 which were obtained from 60 days and 125 days gestation respectively. In both stages of development extensive gap junctions were associated with the junctional network which also exhibited some previously undescribed wide 'complex' strands. More than 120 different tight junctions from various stages of development were examined with an improved freeze-fracture technique (see Material and methods) and the number of strands, subdivided into normal and 'complex' strands was recorded. The proportion of 'complex' and normal strands was rather variable in different junctions at the same foetal age but there was no consistent difference in the proportion at different foetal ages.

In single replicas (Figs. 1 and 2) it was not possible to resolve the fine structure of 'complex' strands although it was obvious that they often contained particulate material in the middle of the P face ridges. However, complementary replicas of these tight junctions demonstrated that the 'complex' strands correspond to two parallel ridges which enclose one row of small particles on the P face, complementary to two parallel grooves which line one row of pits on the E face (Figs. 3A and B, 4A and B). Occasionally, segments of the row of particles separated with E face, which thus appeared as two parallel grooves lining one row of particles. Sometimes a 'complex' strand on the P face continued into a 'normal' strand. The complementary E face exhibited a single row of pits between two grooves corresponding to the 'complex' strand which continued directly into a 'normal' single groove (Figs. 4A and B).

The extensive distribution of gap junctions within tight junction networks was especially evident when complementary fracture faces were examined. E face pits were often difficult to identify, whereas the corresponding P face particles could be easily recognized (Figs. 3A and B). Gap junction particles were frequently completely sequestered within compartments of the tight junction network which were connected to other gap junction-containing compartments via 'complex' strands (for example, Fig. 1). The possible interrelationship between the single row of particles


1
60D
2
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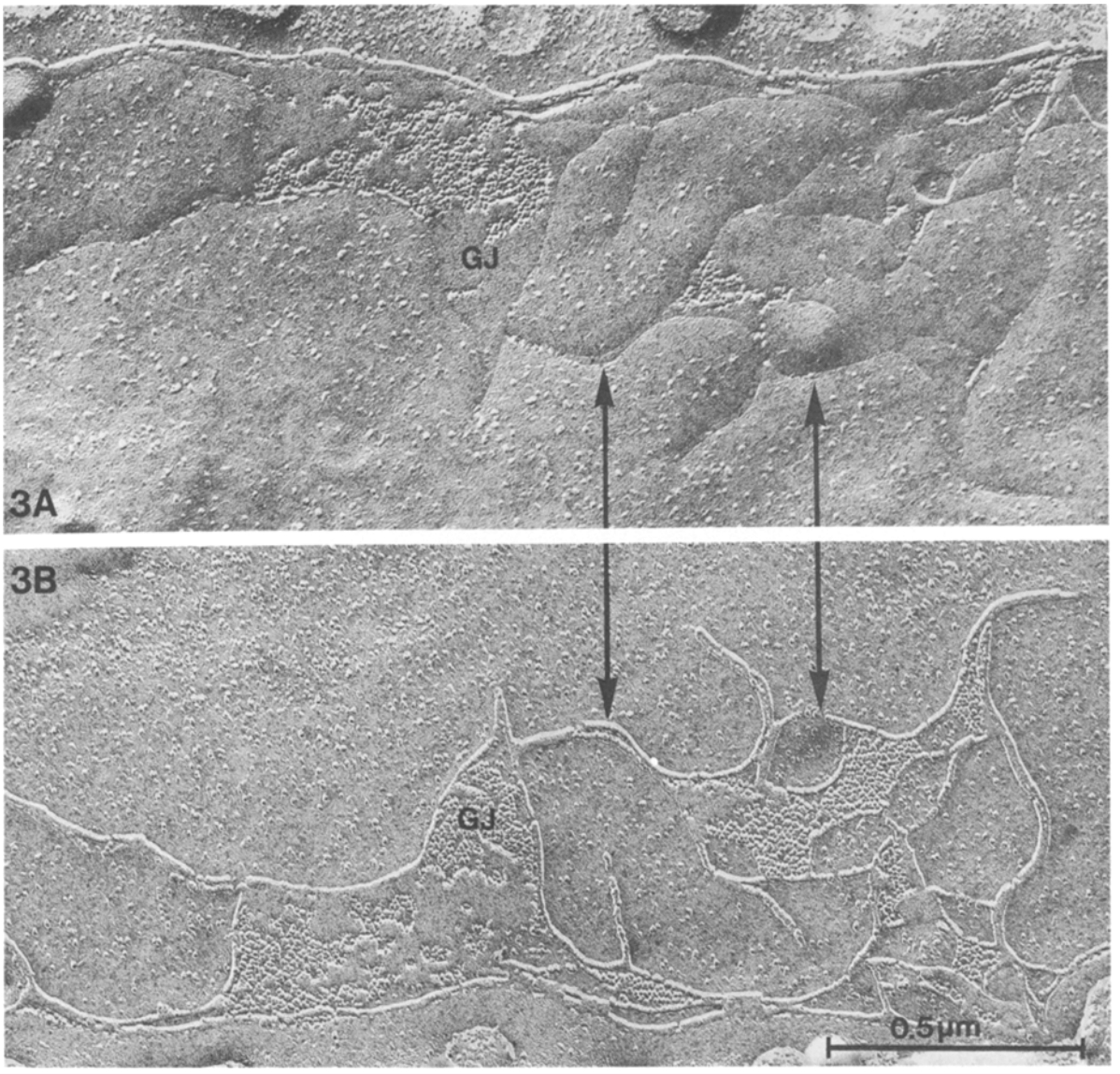
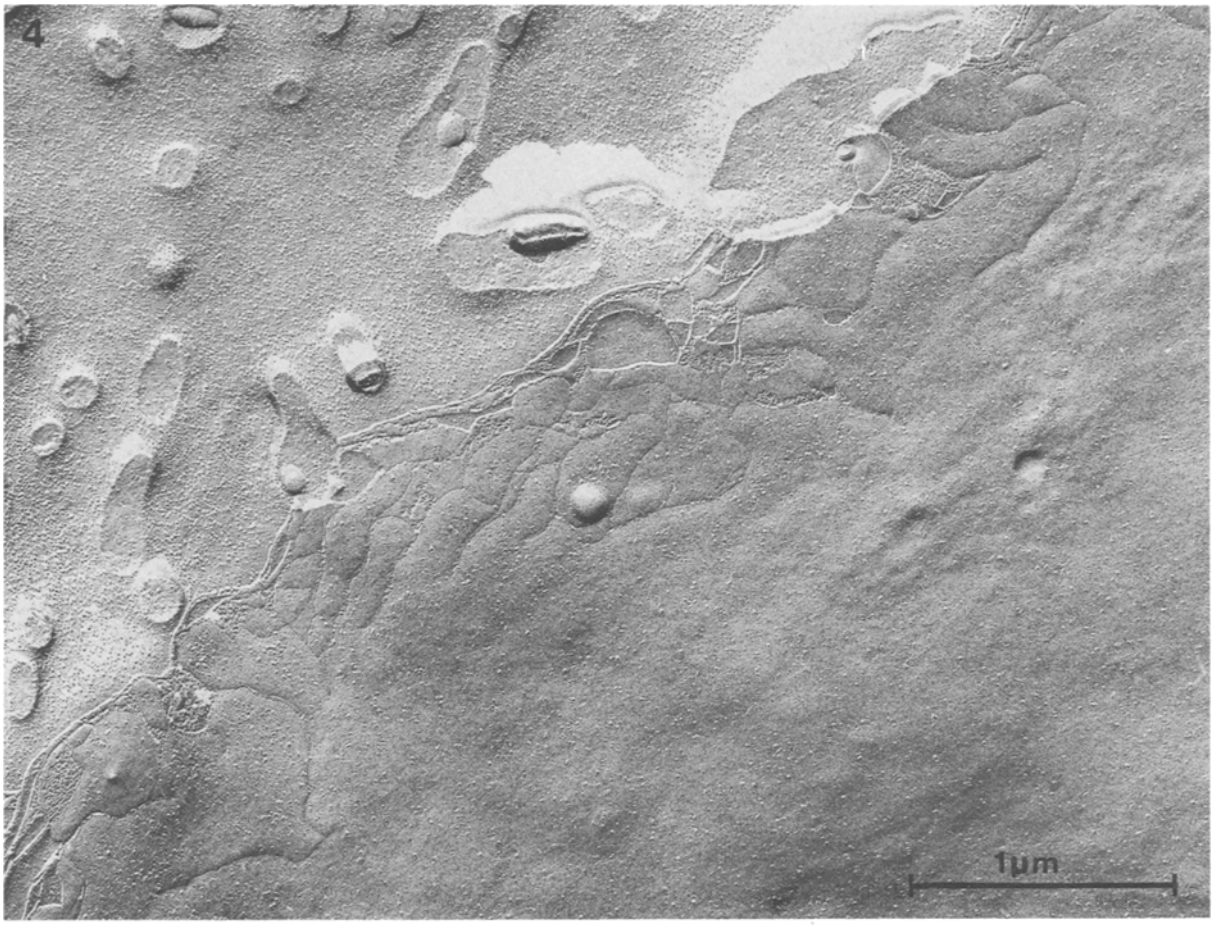


Fig. 3A and B. Complementary membrane halves of the tight junction from 60 days choroid plexus obtained by the double replica technique. Arrows point to complementary areas of particular interest. The double arrows in the middle of the plate demonstrate that the two grooves on the E face (3A) correspond to the two ridges on the P face (3B). Towards the left they fuse to form a 'complex' strand. The right double arrow indicates a particle in the E face groove which corresponds to a small discontinuity in the P face ridge. Note the complementarity of the gap junctions (GJ).

Figs. 1 and 2. Freeze-fracture replicas of choroid plexus epithelial cells from 60 days (Fig. 1) and 125 days (Fig. 2) gestation foetal sheep. The complexity of the well formed tight junction in the early stage (60 D) when the blood–C.S.F. barrier is highly permeable should be compared with the more simple construction of the tight junction at the later and less permeable stage (125 D). At both stages the 'complex' strands form part of the tight junction network. The gap junctions are characteristically enclosed within compartments of the tight junction network in the early stage. Note that some 'complex' strands connect or partially border such compartments. Scale bar: 0.5 µm.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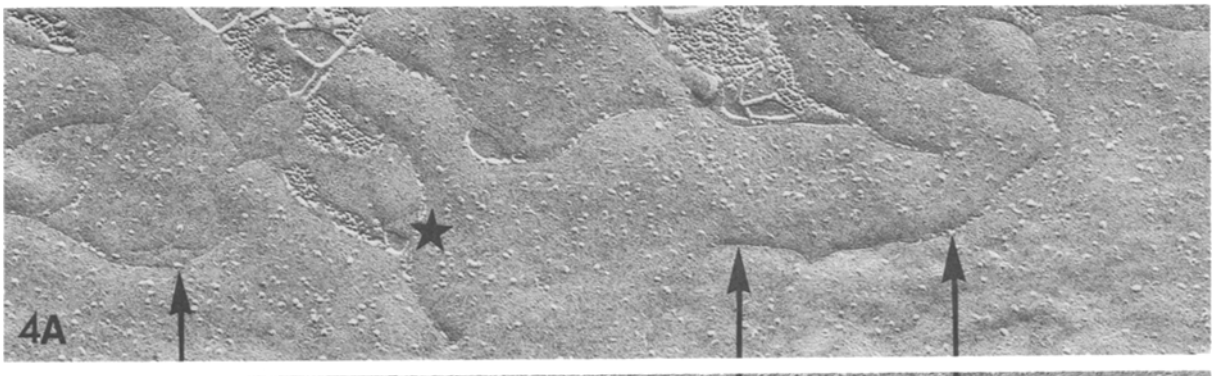
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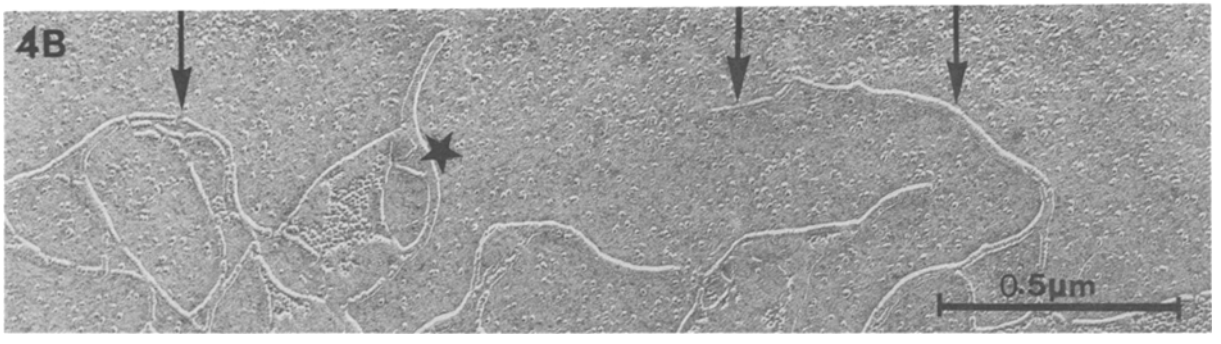
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in the 'complex' strands and gap junction particles is an interesting but unsolved problem.

The fracture procedure usually caused complete separation of the tight junction strands at all gestational ages examined (30–125 days), so that the P face exhibited complete ridges and the E face exhibited particle-free grooves (see Figs. 1 and 2). The very few discontinuities in P face ridges could however be accounted for since the complementary E face grooves contained the missing fragments (Figs. 4 A and B). Based on a thorough examination of 11 complementary pairs of tight junction networks from 45 and 60 days of gestation we suggest that discontinuities in these choroid plexus tight junction ridges are caused by displacement of material during fracturing and are not indicative of incomplete junction formation.

Discussion

The blood–brain and blood–C.S.F. barriers to protein in the adult have clearly been shown to be due to the fact that the junctions between the cerebral endothelial cells and the choroid plexus epithelial cells are tight and because no significant amount of protein crosses these cells (Reese and Karnovsky, 1967; Brightman, 1968; Brightman and Reese, 1969). It has been suggested by several authors (for example, Doolin and Birge, 1969; Delorme, 1972; Birge *et al.*, 1974) that the intercellular tight junctions are incomplete in the foetus and that intercellular penetration of plasma proteins into C.S.F. could account for the high concentration of protein found in foetal C.S.F.

The development of tight junctions has recently been examined with freeze-fracturing in a variety of epithelia (Montesano *et al.*, 1975; Elias and Friend, 1976; Hull and Staehelin, 1976; Porvaznik *et al.*, 1976; Dermietzel *et al.*, 1977). It appears that individual intramembranous particles are arranged into linear segments that fuse to become short linear ridges. These short ridges in turn fuse with other ridge segments to form anastomosing networks that eventually become complete belts.

Fig. 4. Figs. 4A and B are enlargements of complementary fracture faces of the upper right portion of the tight junction shown in Fig. 4. Fig. 4A demonstrates mainly an E face, and Fig. 4B shows the corresponding P face. Arrows (left) demonstrate that the missing particle in the P face ridge is sitting in the corresponding E face groove. Asterisks indicate the two fracture faces of a 'complex' strand, but the best illustration of the differences in freeze-fracture pattern between normal and 'complex' strands is indicated by the two double arrows (right). The pair of arrows near the free end of the strand show the complementary fracture faces of a normal strand. Towards the main portion of the tight junction network the structure of the strand changes. The more than double width of the P face ridge corresponds to a single row of pits lined by two furrows (right hand set of arrows). Compare with Fig. 1 which has a more optimal shadowing angle and particularly in its right half shows clearly the double ridges and furrows enclosing particulate material which are characteristic of 'complex' strands.

When the belt is completed, morphological marker substances are excluded and the permeability barrier is formed (Ducibella *et al.*, 1975; Larsson, 1975).

In chick embryos the first choroidal *anlage* appears at 5–6 days gestation and already on day 6 a tight junction network is present (Dermietzel *et al.*, 1977). Gap junctions develop within the network from day 9 to day 11, by which time 'mature' intercellular junctional complexes have developed.

In our studies on development of the blood–brain barrier we have not yet gone far enough back in development to reach a stage with incomplete tight junctions. The *anlage* of the choroid plexus in foetal sheep probably appears at about day 20; it is present in a section of Åstrom (1967) taken through the brain of a sheep foetus of approximately this gestational age. The earliest stages we have examined so far in freeze-fracture studies of foetal choroid plexus and blood vessels is 30 days gestation (Møllgård and Saunders, 1975; Møllgård *et al.*, 1976, 1977, in preparation). The tight junctions were already well formed at this early age and there were no major changes over a wide range of foetal ages in the freeze-fracture characteristics (especially the number of strands in the network) previously suggested to be related to epithelial permeability (Claude and Goodenough, 1973). This was in spite of marked changes in permeability of the blood–brain and blood–C.S.F. barriers in the same foetal period.

In a search for other structural features of the tight junction network which might be correlated with the observed changes in permeability we have probed further into two additional characteristic aspects of choroid plexus epithelial cell tight junctions – namely the occurrence of 'complex' strands and junctional discontinuities.

Since the proportion of 'complex' strands to the total strand number did not appear to change consistently during the developmental stages investigated so far it is unlikely that this strand variety has any specific significance in determining the transepithelial permeability. Their association with gap junctions is, however, remarkable and one row of gap junction particles would just fit into the interspace between the two parallel strands which line a 'complex' strand. It may well turn out that gap junction particles are inserted in the cell membrane at specific sites in the tight junction network via a system of tubulo-cisternal endoplasmic reticulum–the TER-system (Møllgård and Saunders, 1977; Møllgård and Rostgaard, 1978). Such a process would require a synchronous activity in the TER-systems of adjacent cells as well as some symmetrically aligned contact points between the adjacent TER-cell membrane-cell membrane-TER-systems for which we have preliminary evidence (Møllgård, Rostgaard and Saunders, unpublished). From their points of insertion, pairs of gap junction particles might be distributed to other tight junction regions and later to areas of both lateral and apical cell membranes via the corridors of the 'complex' strands.

Alternatively the 'complex' strands might be involved in tight junction development so that the material between the two parallel strands is utilized for growth and elongation of strands in the tight junction network. This requires investigation in

still younger sheep foetuses. The presence and characteristic structure of 'complex' strands have not been commented upon by previous authors although they can be easily recognized in many published electron micrographs of freeze-fractured tight junctions, thus indicating that this strand variety is widely distributed among various epithelial tissues and therefore not confined to the foetal choroid plexus.

In purely descriptive terms junctional discontinuities might be interesting in their own right, but in combined morphological and transport physiological studies it is crucial to know whether discontinuity in a junctional strand exemplifies an incomplete separation of junctional material during the freeze fracture process or whether it represents an absence of junctional material and thus indicates a genuine pathway through the strand in question. In our earlier studies without the double replica technique we observed only a few discontinuities in the P face ridges and occasional particles in the E face grooves which prompted us to believe that the discontinuities were caused by the fracture process. Also we found approximately the same number of discontinuities in early and late stages of development indicating that the phenomenon could not be correlated with the changes in permeability.

In the present study we have demonstrated by the double replica technique that discontinuities at least in foetal sheep choroid plexus are caused by displacement of material during the fracture process and therefore are not indicative of incomplete junction formation. A very recent study on the development of another barrier, the alveolar barrier, between blood and lung liquid in sheep foetuses also reached a similar conclusion using the double replica technique (Schneeberger *et al.*, 1978).

The findings of displaced junctional material together with the constant occurrence of 'complex' strands lend support to the hypothesis that the marked changes in permeability of the blood–C.S.F. barrier during the development cannot be accounted for by changes in the morphology of the paracellular tight junction route. The high permeability is more likely to reflect the presence of a transcellular route, probably via the previously described TER-system.

Acknowledgements

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